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Original Article

Involvement of V-Ets erythroblastosis virus E26 oncogene homolog 2 in regulation of transcription activity of *MDR1* gene

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Over-expression of *MDR1* confers multidrug resistance (MDR) in cancers and remains a major cause for the failure of chemotherapy. In the present study, we found that V-Ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) could activate *MDR1* transcription and P-glycoprotein (P-gp) expression in SGC7901 cells. Knockdown of ETS2 attenuated *MDR1* transcription and P-gp expression, and increased the sensitivity of MDR cancer cells to cytotoxic drugs that were transported by P-gp in SGC7901/VCR cells. ETS2 could bind to the ETS2 sites on the *MDR1* promoter and activate its transcription. The regulation of *MDR1* expression by ETS2 may provide potential ways to overcome MDR in cancer treatment.

Keywords multidrug resistance; ETS2; *MDR1*

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Introduction

The *MDR1* (ABCB1) gene is located on chromosome 7 and consists of 28 exons. Its product P-glycoprotein (P-gp) is the best studied member of the ATP-binding cassette (ABC) family of transporters [1,2]. P-gp is an efflux pump that is known to confer resistance to a variety of clinically important antineoplastic agents, including doxorubicin, vincristine, taxanes, etoposide, teniposide, and actinomycin D. Many of these agents are used to treat cancers [3,4]. Over-expression of *MDR1* in cancer confers MDR and remains a major cause for the failure of chemotherapy.

Transcription of *MDR1* can be induced or repressed by a variety of transcriptional factors. Nuclear transcription factor Y, Sp1 transcription factor and Y box-binding protein I play important roles in the transcriptional activation of the *MDR1* gene by genotoxic stress [5–7]. Histone methyltransferase myeloid/lymphoid or mixed-lineage leukemia 1 (MLL1) increases *MDR1* transcription and chemoresistance, while p53 or ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1) inhibits *MDR1* gene transcription and sensitizes cancer cells to anticancer drugs [8–10]. MyoD or ets variant 4 (PEA3) could bind to the E-box and PEA3 sites on the *MDR1* promoter and activate its transcription [11].

V-Ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) is a transcription factor, and so far, there is no report as to whether ETS2 can regulate *MDR1* transcription. The aim of this study was to explore the activity of ETS2 on *MDR1* transcription and to reveal the role of ETS2 involved in *MDR1*-mediated drug resistance, which would be helpful to understand the MDR mechanism.

Materials and Methods

Cell lines and plasmids

The MDR gastric cancer cell line, SGC7901/VCR, and the sensitive cell line, SGC7901, were purchased from Wuhan University Type Culture Collection (Wuhan, China). They were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. For SGC7901/VCR cell line, 0.8 μmol/l of vincristine was added to the medium for the maintenance of the MDR phenotype.

MDR1 promoter/luciferase construct pGL2-MDR1 (−1202 to +118) was kindly provided by Dr Kathleen W. Scotto (Cancer Institute of New Jersey, New Brunswick, USA). ETS2 expression vector was purchased from Origene Company (Rockville, USA).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cells or tissue with TRIzol reagent (Invitrogen). The reverse transcription (RT) reaction was performed using a Superscript First-Strand Synthesis System (Invitrogen). The newly synthesized

cDNA was amplified by polymerase chain reaction (PCR). The reaction mixture contained 2 μl of cDNA template, 1.5 mM $MgCl_2$, 2.5 units of *Taq* polymerase, and 0.5 μM primers. Genes and primers are listed as follows: *MDR1*, forward primer 5′-ATATCAGCAGCCCACATCAT-3′, reverse primer 5′-GAAGCACTGGGATGTCCGGT-3′; *ETS2*, forward primer 5′-GTGGACCTATTCAGCTGTG G-3′, reverse primer 5′-TTCCCCGACGTCTTGTGGAT-3′. *GAPDH* was used as an internal control, the primers are as follows: 5′-GCCAAAAGGGTCATCATCTC-3′ and 5′-GT AGAGGCAGGGATGATGTTC-3′. Amplification cycles were: 94°C for 3 min, then 33 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, followed by 72°C for 10 min. Aliquots of PCR product were subject to electrophoresis on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

Western blot analysis

Cells were washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride, and lysed in mammalian protein extraction buffer (Pierce, Rockford, USA). The lysates were transferred to eppendorf tubes and clarified by centrifugation at 12,000 *g* for 40 min at 4°C. Identical amounts (50 μg of protein) of cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose (Millipore, Billerica, USA). The membranes were incubated in blocking solution consisting of 5% powdered non-fat milk in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, then immunoblotted with anti-P-gp antibody C219 (1 : 1000; Calbiochem, San Diego, USA), anti-ETS2 antibody (1 : 200; Santa Cruz, Santa Cruz, USA), or anti-GAPDH antibody (1 : 3000; Sigma-Aldrich, St Louis, USA) overnight at 4°C, respectively. After being washed three times with Tris-buffered saline and Tween 20 (TBST), the membranes were incubated with horseradish peroxidase-conjugated secondly antibodies for another 1 h followed by three times wash with TBST and then developed by enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech, Piscataway, USA) according to the manufacturer's instructions.

Small interfering RNA preparation and transfection

The small interfering RNA (siRNA) targeting ETS2 and non-targeting siRNA were purchased from Shanghai GeneChem (Shanghai, China). The target sequence for ETS2 is 5′-CAACAGGCUUGGAUUCCAU-3′. Cells in the exponential phase of growth were seeded in six-well plates at a concentration of 5×10^5 cells/well. After incubation for 24 h, the cells were transfected with siRNA specific for ETS2 or non-targeting siRNA at a final concentration of 100 nM using oligofectamine and OPTI-MEMI-reduced serum medium (Invitrogen), according to the manufacturer's protocol. Silencing efficiency was examined at 48 h after transfection.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer's protocol (Active motif, Carlsbad, USA). Briefly, cells in 150 mm tissue culture dishes were fixed with 1% formaldehyde and incubated for 10 min at 37°C. The cells were then washed twice with ice-cold PBS, harvested, and resuspended in ice-cold TNT lysis buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 1% aprotinin). The lysates were sonicated to shear the DNA to fragments of 200–600 bp, and subjected to immunoprecipitation with ETS2 antibody or IgG (Santa Cruz) as negative control. Three micrograms of antibody was used for each immunoprecipitation. The antibody/protein complexes were collected by Protein G beads and washed three times with ChIP wash buffer (5% SDS, 1 mM EDTA, 0.5% bovine serum albumin and 40 mM $NaHPO_4$, pH 7.2). The immune complexes were eluted with 1% SDS and 1 M $NaHCO_3$, and the cross-links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl and RNase A. The samples were then treated with proteinase K for 2 h, and DNA was purified by mini-column, ethanol precipitation, and resuspended in 100 μl of H_2O. The primers corresponding to the *MDR1* promoter region −164 and −11 upstream of the transcription start site (sense: 5′-CCTCCTGGAAATTCAACCT G-3′; antisense: 5′-TGTGGCAAAGAGAGCGAAG-3′) was used for PCR to detect the presence of the *MDR1* promoter DNA.

Mutagenesis

MDR1 promoter/luciferase construct pGL2-MDR1 was used as template. The plasmid DNA was methylated with DNA methylase at 37°C for 1 h. The plasmid was amplified in a mutagenesis reaction with two overlapping primers, one of which contains the target mutation. The product was linear, double-stranded DNA containing the mutation. The mutagenesis mixture was transformed into wild-type *Escherichia coli*. The host cells circularized the linear mutated DNA, and McrBC endonuclease in the host cells could digest the methylated template DNA, only leaving the unmethylated and mutated product. For individual mutations, the sequence of ETS2 binding site (ETS2-1, ETS2-2, and ETS2-3) AGGAA was converted to ATATG.

Luciferase reporter gene assay

SGC7901 cells were seeded in six-well plates at a density of $1–2 \times 10^5$ cells/well and cultured for 24 h. Cells were then co-transfected with wild-type (pMDR1) or mutants (ETS2 mutant) *MDR1* reporter construct (0.5 μg/well) and 0.5 μg of pcDNA3 or ETS2 expression vector, together

with 20 ng of control Renilla luciferase reporter construct pRL-TK (Promega, Madison, USA). The total amount of DNA per well was adjusted to 1.5 μg by the addition of sonicated salmon sperm DNA. Luciferase assays were performed as recommended by the vendor (Promega) and normalized relative to protein concentration as determined by the bicinchoninic acid protein assay (Pierce). The promoter activity was then expressed as luminescence units, which is the ratio of luminescence counts of cell lysate and the absorbance at 595 nm for the same amount of cell lysate stained with bicinchoninic acid protein assay reagent.

Drug sensitivity assay

SGC7901/VCR cells transfected with ETS2 siRNA or non-targetting siRNA (control siRNA) were plated in 96-well plates in growth medium and incubated at 37°C in a humidified 5% CO_2 atmosphere for 60 h in the presence of varying concentrations of vincristine, doxorubicin, paclitaxel, and cisplatin. At the end of incubation, the viability of cells was measured using the MTT assay. Briefly, 20 μl metrizamide (5 mg/ml, Sigma) was added to each well, and the cells were continuously cultured for 4 h. The supernatant was removed and 150 μl dimethyl sulfoxide was added to each well and agitated for 10 min to fully liquefy crystals. Optical density was detected at 490 nm by an automatic enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek, Burlington, USA).

Statistical analysis

Analysis of variance and Student's *t*-test were used to determine the statistical significance of differences between experimental groups. P values <0.05 were considered significant, and the confidence intervals quoted were at the 95% level.

Results

Binding status of ETS2 on the *MDR1* promoter between SGC7901 and SGC7901/VCR cells

In this study, we found that the *MDR1* transcription and P-gp expression were dramatically increased in SGC7901/VCR cells compared with in SGC7901 cells [**Fig. 1(A,B)**]. In order to determine the potential transcriptional factors binding to the *MDR1* promoter, SGC7901 and SGC7901/VCR cells were subject to ChIP assays. The results demonstrated that ETS2 could bind directly to the transcriptional *MDR1* promoter in SGC7901/VCR cells [**Fig. 1(C)**].

Over-expression of ETS2 increases *MDR1* transcription and P-gp expression in SGC7901 cells

To identify the role of ETS2 in regulating the expression of P-gp, we transfected ETS2 expression vector or control vector into SGC7901 cells, respectively. **Figure 2(A)** showed that the over-expression of ETS2 could increase *MDR1* transcription in SGC7901 cells. **Figure 2(B)** showed that the over-expression of ETS2 could increase P-gp expression in SGC7901 cells. ChIP results demonstrated that the over-expression of ETS2 increased the presence of ETS2 on the *MDR1* promoter [**Fig. 2(C)**].

Effect of ETS2 on the *MDR1* promoter activity

To identify the role of ETS2 in regulating *MDR1* promoter transcription, we co-transfected the *MDR1* promoter/luciferase construct pMDR1 with pcDNA3 or ETS2 expression vectors into SGC7901 cells. **Figure 3(B)** showed that the luciferase activity were enhanced significantly by ETS2, indicating that ETS2 were involved in the activation of the *MDR1* promoter activity.

We found four ETS2 sites from −200 to +1 bp on the *MDR1* promoter, as shown in **Table 1** and **Fig. 3(A)**. To determine the potential roles of these ETS2 elements in regulation of *MDR1* gene transcription, we individually and combinatorially mutated the three ETS2 sites (ETS2-1, ETS2-2, and ETS2-3) which are close to the transcriptional start site on the *MDR1* promoter, and examined ETS2-inducible reporter gene activities in SGC7901 cells. As shown in **Fig. 3(C)**, any single mutation of three ETS2 sites decreased the reporter gene activity compared with wild-type *MDR1* promoter construct. Mutations of any two

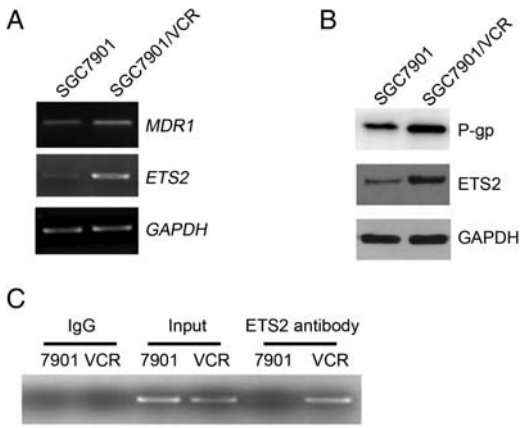

Figure 1 Binding status of ETS2 on the *MDR1* promoter between SGC7901 and SGC7901/VCR cells (A) Relative *MDR1* mRNA level was detected in SGC7901 and SGC7901/VCR cells. Total RNA was extracted from the cells, and RT-PCR was performed. (B) P-gp protein level was detected in SGC7901 and SGC7901/VCR cells. Cell lysates were prepared from SGC7901 and SGC7901/VCR cells, and equal amounts (50 μg) of proteins were resolved by SDS-PAGE. P-gp was detected by immunoblotting with anti-P-gp antibody. GAPDH was used as a loading control. (C) Binding status of ETS2 on the *MDR1* promoter in SGC7901 and SGC7901/VCR cells. Nucleic extracts were prepared from SGC7901 and SGC7901/VCR cells. ChIP assays were performed using antibody against ETS2. The primers corresponding to the *MDR1* promoter region −164 and −11 upstream of the transcription start site were used for RT-PCR to detect the presence of the *MDR1* promoter DNA.

ETS2 sites simultaneously caused a further decrease of reporter gene activity, and the combined mutations of all three ETS2 sites resulted in the maximal decrease of reporter gene activity. These results suggest that all three ETS2 sites contribute in a concerted mechanism to the ETS2-induced transcription of *MDR1* gene, and ETS2 can activate *MDR1* transcription by binding to a number of ETS2 sites on the *MDR1* promoter.

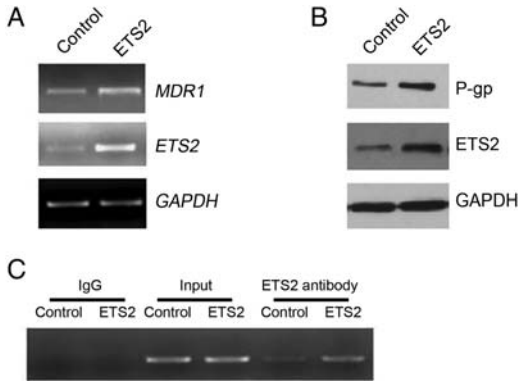

Figure 2 Overexpression of ETS2 increases P-gp expression in SGC7901 cells SGC7901 cells were transfected with control vector or ETS2 expression vector for 48 h. RT-PCR (A) and western blot (B) analysis were performed as described in 'Materials and Methods' section. GAPDH was used as a control. (C) Binding status of ETS2 on the *MDR1* promoter in SGC7901 cells transfected with control vector or ETS2 expression vector. Nucleic extracts were prepared from cells. ChIP assays were performed using antibody against ETS2. The primers corresponding to the *MDR1* promoter region −164 and −11 upstream of the transcription start site were used for RT-PCR to detect the presence of the *MDR1* promoter DNA.

Knockdown of ETS2 attenuates *MDR1* expression, presence of ETS2 on the *MDR1* promoter

SGC7901/VCR cells were treated with 100 nM of ETS2 siRNA or non-targeting siRNA for 48 h. RT-PCR and western blot analysis were performed. As shown in **Fig. 4(A,B)**, ETS2 siRNA significantly inhibited the expression of ETS2 mRNA and protein in SGC7901/VCR cells after transfection for 48 h. At the same time, we found that knockdown of ETS2 inhibited *MDR1* mRNA and P-gp protein significantly. ChIP results demonstrated that knockdown of ETS2 inhibited the presence of ETS2 on the *MDR1* promoter [**Fig. 4(C)**].

Knockdown of ETS2 can modulate MDR phenotype

In above studies, we found that ETS2 could bind to ETS2 sites on the *MDR1* promoter and activate its transcription, and the over-expression of ETS2 could increase P-gp expression, knockdown of ETS2 could attenuate *MDR1* transcription and P-gp expression. We next tested whether knockdown of ETS2 could increase the sensitivity of MDR cancer cells to cytotoxic drugs that were transported by P-gp. SGC7901/VCR cells were transfected with siRNA targeting ETS2 or non-targetting siRNA for 6 h, and then were seeded in 96-well plates and incubated in the presence of various concentrations of doxorubicin, paclitaxel, vincristine or cisplatin for 54 h. As shown in **Table 2**, compared with control MDR cells, the sensitivity of MDR cells

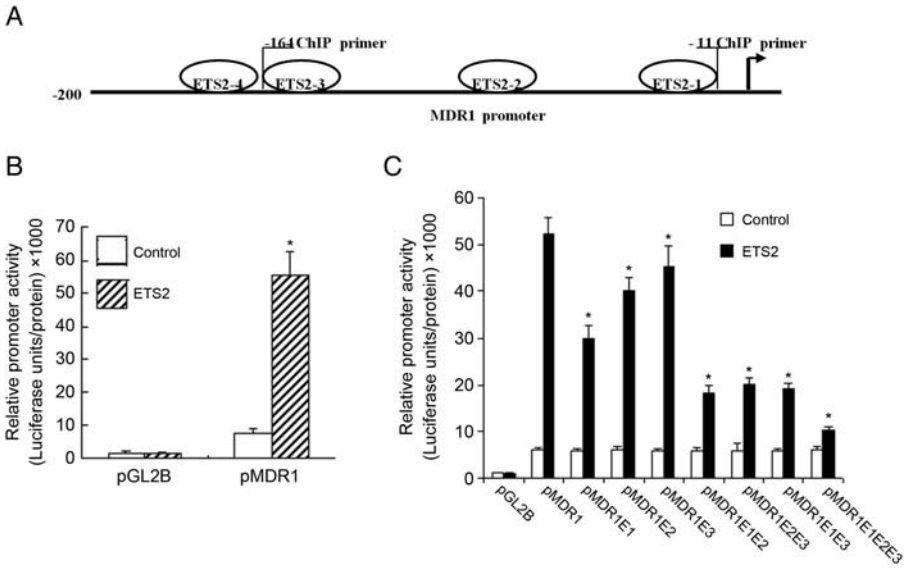

Figure 3 Effect of ETS2 on *MDR1* promoter activity (A) Model depicting the ETS2 sites on the *MDR1* promoter. (B) Activation of the *MDR1* promoter by ETS2. Luciferase assays were performed as described in 'Materials and Methods' section. $*P < 0.05$ versus controls. (C) Effects of mutations of ETS2 sites on the *MDR1* promoter on ETS2-induced promoter activities in SGC7901 cells. Single or combined mutations of ETS2-1, ETS2-2 and ETS2-3 sites were separately made and luciferase assays were performed as described in 'Materials and Methods' section. $n = 3$, $*P < 0.05$ versus controls.

Table 1 ETS2 sites on the MDR1 promoter

No.	MDR1 promoter	
	Region (bp)	Site sequence
1	−11 to −15	AGGAA (ETS2-1, E1)
2	−14 to −68	AGGAA (ETS2-2, E2)
3	−129 to −133	AGGAA (ETS2-3, E3)
4	−166 to −170	TTCCT (ETS2-4, E4)

transfected with ETS2 siRNA to doxorubicin, vincristine, and paclitaxel was increased significantly. However, the sensitivity to cisplatin, a drug that is not transported by P-gp, was not affected by silencing of ETS2 expression. As shown in **Fig. 5**, the intracellular accumulation of doxorubicin was increased in ETS2-siRNA-treated SGC7901/VCR cells, as compared with that in non-targeting siRNA-treated cells.

Discussion

Erythroblast transformation-specific (ETS) family is a large family with at least 30 members that function as transcription factors. All ETS transcription factors share a highly conserved DNA-binding domain, the ETS domain [12,13]. ETS2, a member of the ETS family, has been shown to interact with zinc finger, Myleoid, Nervy and DEAF-1 (MYND) domain containing protein 11, cyclin-dependent kinase 10, c-jun, and myb to regulate target gene expression [14–17]. ETS2 is a proto-oncogene and involved in growth, transformation, and apoptosis. Over-expression of ETS2 can transform primary fibroblasts and induce tumor in the nude mice, which indicated that ETS2 can regulate cell proliferation and transformation [18–20]. ETS2 activates or represses the transcription of target genes in a context-dependent manner [21]. Elevated expression of ETS2 has been correlated with human breast cancer [22]. ETS2 deletion in tumor-associated macrophages can decrease the frequency and size of lung metastases in three different mouse models of breast cancer metastasis. ETS2 ablation in tumor-associated macrophages can lead to decreased angiogenesis and decrease growth of tumors [23].

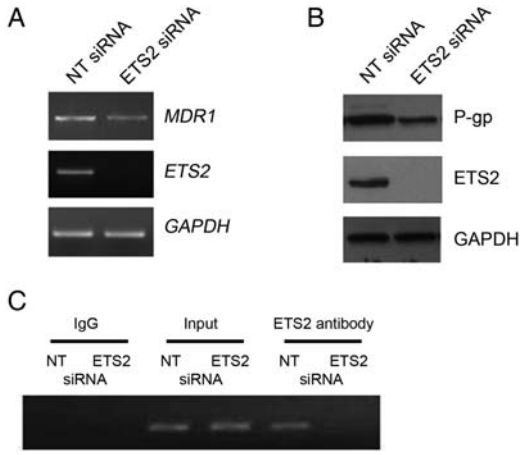

Figure 4 Knockdown of ETS2 attenuates *MDR1* expression in SGC7901/VCR cells SGC7901/VCR cells were treated with ETS2 siRNA or non-targeting siRNA for 48 h, RT-PCR (A) and western blot (B) analysis were performed as described in 'Materials and Methods' section. (C) Binding status of ETS2 on the *MDR1* promoter in SGC7901/VCR cells transfected with non-targeting siRNA and SGC7901/VCR cells transfected with ETS2 siRNA. Nucleic extracts were prepared from cells. ChIP assays were performed using antibody against ETS2. The primers corresponding to the *MDR1* promoter region −164 and −11 upstream of the transcription start site were used for RT-PCR to detect the presence of the *MDR1* promoter DNA.

We used SGC7901 and SGC7901/VCR cells in this study. SGC7901/VCR is an established VCR-resistant cell line selected by stepwise exposure of parental SGC7901 cells to increasing concentrations of VCR [24]. Previous reports have proved that P-gp expression is very low in SGC7901 cells, but high in SGC7901/VCR cells. The SGC7901/VCR cell line was 45 times more resistant to VCR than the parental SGC7901 cell line [25]. The SGC7901/VCR cell line has been successfully used as an *in vitro* MDR reversal model [26].

In this study, we found that *MDR1* expression was low in SGC7901 cells and *MDR1* expression was activated in SGC7901/VCR cells, and the presence of ETS2 significantly increased the transcriptional *MDR1* promoter in SGC7901/VCR cells. Knockdown of ETS2 could inhibit the presence of ETS2 on the *MDR1* promoter region in SGC7901/VCR cells, indicating ETS2 might regulate

Table 2 Effect of knockdown ETS2 on sensitivity of MDR cells to chemotherapeutic drugs

	IC_{50}			
	Doxorubicin (μg/ml)	Paclitaxel (μg/ml)	Vincristine (μ/ml)	Cisplatin (μg/ml)
Non-targetting siRNA	1.76 ± 0.31	169.52 ± 23.66	25.59 ± 3.38	1.12 ± 0.27
ETS2 siRNA	1.03 ± 0.21*	109.12 ± 18.32*	13.32 ± 1.61*	1.02 ± 0.31

IC_{50} is the concentration of the drug that inhibits cell survival by 50%. Results are the mean ± standard error of three independent experiments. *$P < 0.05$ versus non-targetting siRNA-transfected.

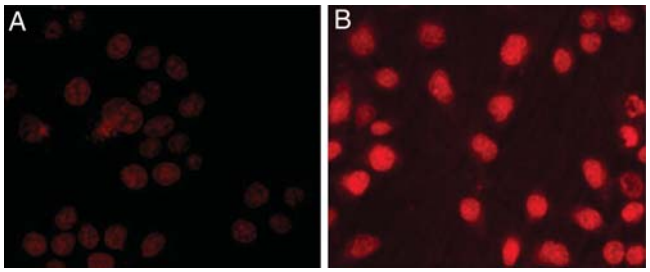

Figure 5 Effect of silencing of ETS2 expression on doxorubicin accumulation in SGC7901/VCR cells SGC7901/VCR cells were treated with ETS2 siRNA or non-targeting siRNA for 48 h, fluorescence microscopy were used to detect the fluorescent signal strength of doxorubicin (10 μg/ml) in non-targeting siRNA transfected SGC7901/VCR cells (A) or ETS2 siRNA transfected SGC7901/VCR cells (B) (×200).

MDR1 expression. Bioinformatic analysis of the 5′-flanking region of the human *MDR1* gene showed that there existed four ETS2 sites on the *MDR1* promoter region from −200 to +1 bp, which indicated that ETS2 might bind to *MDR1* promoter and regulate *MDR1* transcription. Point mutant of ETS2 site in the *MDR1* promoter abrogated the activation effect of ETS2 on *MDR1* promoter activity, indicating that ETS2 activated *MDR1* promoter activity by directly binding to the ETS2 site of *MDR1* promoter.

In this study, we also found that the over-expression of ETS2 could increase P-gp expression, knockdown of ETS2 could attenuate *MDR1* transcription and P-gp expression, which further confirmed that ETS2 was responsible for P-gp expression. Treatment of MDR cells with ETS2 siRNA enhanced intracellular drug accumulation and the sensitivity of MDR cells to doxorubicin, paclitaxel, and vincristine, but had no effect on the non-P-gp substrate drug, cisplatin. All these data demonstrated that ETS2 could regulate the sensitivity of MDR cells by influencing P-gp expression.

We conclude that ETS2 can activate *MDR1* transcription. Knockdown of ETS2 attenuates *MDR1* expression and increases the sensitivity of MDR cancer cells to cytotoxic drugs that are transported by P-gp. ETS2 can bind to ETS2 sites on the *MDR1* promoter and activate its transcription. These are the first studies to our knowledge to describe that ETS2 activates MDR1 expression and ETS2 is a new gene related to MDR. The elucidation of the role of ETS2 in *MDR1* gene transcription might help develop new strategies to inhibit or prevent the induction of *MDR1* expression.

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